Collaborative Study of a Modified Resazurin Test for Estimating Bacterial Count in Raw Maple Sap

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A modified resazurin test technique for rapid estimation of bacterial counts in maple sap was studied in 13 collaborating laboratories. Statistical analysis of the collaborators' results indicates that this method can rapidly identify heavily contaminated sap which would yield low grade sirup or would be unsuitable for sirup manufacture. The method has been adopted as official first action.

The modified resazurin test method for estimating the bacterial count in raw maple sap reported by the Associate Referee in 1971 (1) was studied by 13 collaborating laboratories. The collaborators represented university, industrial, State, and federal governmental facilities. The practical need for the test and its principle were discussed earlier (2).

In anticipation of this study, 2 mixed cultures of natural sap contaminants were secured from sap collected during 2 different late-season sap runs of 1972. The mixed cultures were designated "L" and "S," respectively. Both mixed cultures contained the typical mixture of *Pseudomonas, Aerobacter, Leuconostoc,* and *Bacillus* spp. found in late-season raw maple sap. The cultures were maintained on tryptone glucose agar slants. The slants were incubated at 37.5°C for 24 hr after inoculation and then stored at 5°C. The cultures were transferred at weekly intervals prior to the collaborative study.

The collaborating laboratories were supplied with the following materials: a copy of the test method; a schematic diagram of a suggested method for dilution of the test culture; Munsell standard swatchs showing the control color 7.5 PB6/10 and the end point color 2.5 P6/8; a data sheet for recording test results; 1 pt can maple sirup for simulated sap make-up; standard resazurin dye tablets; one slant culture (L or S) of natural maple sap microorganisms.

The collaborators were instructed to maintain the stock cultures as described above and to perform the test within 8 weeks after receipt of the test materials. A deadline date of February 15, 1973 was specified for reporting data.

Six of the collaborators were sent slants containing the L culture and 7 collaborators received the S culture. They were instructed to prepare 3 randomly contaminated samples of simulated sap made by washing microorganisms of the test culture from a slant which had been incubated 24 hr at 37.5°C and subsequently making serial dilutions 10^{-1} and 10^{-2} of the contaminated simulated sap. This enabled the collaborators to carry out 9 determinations on the samples and their respective dilutions covering a wide range of contamination levels which, it was expected, would exhibit a corresponding variation in the incubation times required for the determinations to reach the positive end point color. The collaborators were also instructed to strive for high bacterial populations (10^7 cells/ml) in 1 or 2 of the samples tested, since the ultimate value of this test would be in its enabling a maple producer to identify raw sap of inferior sanitary quality which would yield an inferior or unwholesome product when evaporated to sirup density. The method used in this study is given below.

METHOD

Bacterial Population of Maple Sap

31.D01 ***Principle***

Resazurin is reduced to resorufin in direct proportion to bacterial action, with color change from purple to pink. Method permits rapid estimation of bacterial populations $>1 \times 10^6$ cells/ml in maple sap.

31.D02 ***Apparatus***

(**a**) *Serological pipets.*—To deliver 1 and 10 ml, with 1.0 ml graduations; sterilized.

(**b**) *Test tubes.*—150 × 16 mm, screw-top with molded plastic caps; sterilized.

Received August 16, 1973.

The mention of commercial items is for convenience and does not constitute an endorsement by the Department of Agriculture.

(c) *Incubator or water bath with opaque cover.*—Constant temp., capable of maintaining 37.5± 0.5°.

(d) *Bottles.*—200 ml amber, g-s.

31.D03 ***Reagents***

(a) *Nonfat milk soln.*—Dissolve 100 g instant nonfat dry milk in 500 ml H_2O. Autoclave 15 min at 121°.

(b) *Resazurin dye.*—Autoclave 200 ml H_2O in amber glass bottle 15 min at 121°. Using sterile forceps, add 1 std (certified by Biological Stain Commission) resazurin dye tablet (Allied Chemical Corp.) and shake to completely dissolve dye before H_2O cools. Store in cool, dark place. Prep. weekly.

(c) *Sterile maple sap control.*—Place 10 ml raw sap in test tube and autoclave 15 min at 121°.

31.D04 ***Technic***

To sterile test tube, transfer 1 ml nonfat milk soln and 10 ml sample. Prep. control tube using 1 ml nonfat milk soln and 10 ml sterile maple sap control. Mix by capping and inverting tubes. Incubate 30 min at 37.5°, using incubator, **31.D02(c)**. Remove tubes and, with sterile pipet, add 1 ml resazurin dye soln to each. Cap tubes, invert to mix thoroly, and incubate at 37.5°. Do not agitate tubes before reading. Examine each tube for color change to Munsell std bluish purple 2.5 P6/8 (Munsell Color Co., Inc., 2441 N. Calvert St., Baltimore, MD 21218) end point at 0.5, 1, 2, 3, 4, and 5 hr. Color control should match Munsell std 7.5 PB6/10 and should remain stable for duration of test. Calc. bacterial cell population from: $\log Y = 7.84 - 0.587\ X$, where Y = bacterial count, cells/ml, and X = time for color development, hr.

Results and Recommendation

Collaborators' results were tabulated and curves were constructed in which the logarithms of the bacterial populations in the sap samples were plotted vs. the time required for these microorganisms to reduce the resazurin dye to the Munsell 2.5 P6/8 end point specified in the method. Figure 1 shows a curve constructed from 42 determinations submitted by collaborators who used the L culture. The solid line was fitted by the least squares method, and the interrupted lines delineate 95% confidence limits. An analysis of variance of these data showed a significant F-value, 144.08 ($p = <0.01$). Figure 2 shows an identical treatment of data for 62 determinations received from collaborators using the S culture.

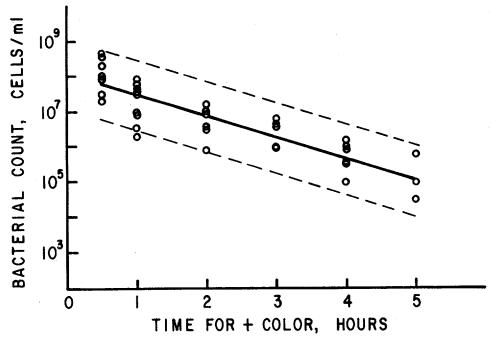

FIG. 1—Relation of L culture bacterial populations in simulated maple sap to resazurin dye reduction times, plotted on semilog scale.

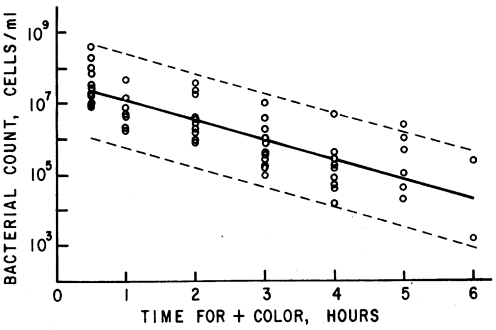

FIG. 2—Relation of S culture bacterial populations in simulated maple sap to resazurin dye reduction times, plotted on semilog scale.

The analysis of variance for these data showed an F-value of 155.52 ($p = <0.01$). The curves of these 2 figures show an excellent agreement in the slopes of the lines (L culture = −0.604, S culture = −0.557) and are also similar to the original curve reported at the 1971 Annual Meeting of the AOAC (1). In each figure, only 1 determination reported by a collaborator lay beyond the delineation of the 95% confidence limits.

Figure 3 shows a curve constructed in the same manner as Figs. 1 and 2 from the combined data of all collaborators. An analysis of variance of the combined data showed an F-value of 238.74 ($p = <0.01$). The slope of this line is −0.586. Only 2 of the collaborators' determinations lay beyond the 95% confidence limits; a "high count" positive reaction at 5 hr incubation time and a "low count" at 4 hr. In a practical application of this test, neither result would be of significance to a maple producer intent upon segregating heavily

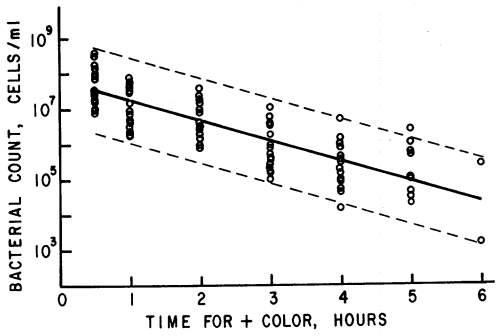

FIG. 3—Plot of combined collaborators' data—L and S culture bacterial populations vs. resazurin reduction times, plotted on semilog scale.

contaminated lots of sap from normal sirup production lines. It is also noteworthy that all of the data showing high bacterial counts requiring 3, 4, and 5 hr incubation before positive color reactions were obtained were submitted by the collaborator farthest removed from the Associate Referee's Laboratory. It is possible that the mixed culture sent to this collaborator lost much of its vigor when subjected to the rigors of shipment over a long distance in the early winter season.

A compilation of the data from which the graphs were constructed is given in Table 1. Data from 1 collaborator who used the L culture are not shown in Table 1 because this collaborator reported a positive color reaction in all tubes including the control after 30 min incubation. This was probably due to failure to dissolve the standard dye tablet completely. In our laboratory we found that a weak resazurin solution resulting from failure to dissolve the dye tablet completely produced a false positive color immediately upon its addition to the sap/milk solution. It is possible that a highly contaminated milk solution could produce the same false positive reaction, although it is most unlikely that this was the cause of the reaction observed by this collaborator, since freshly reconstituted nonfat dry milk is quite low in bacterial count.

Collaborators' comments were quite favorable regarding the method. Two collaborators reported that they could merely use the color standards as an approximate guide, because in sterilizing the nonfat milk, they encountered a heat-induced browning reaction. Nevertheless, these collaborators submitted data which were among the most concordant results received by the Associate Referee.

This method was put to a practical test by J. L. Sipple of the J. L. Sipple Central Evaporator Plant, Bainbridge, N.Y., during the 1973 maple season. Mr. Sipple performed the tests personally and commented that sap which reduces the dye in over 3 hr yields light amber sirup, sap showing a positive color reaction in 2 hr yields medium amber sirup, and sap which reduces the dye in 1 hr yields commercial grade sirup. A ½ hr or less positive color indicated a sap of such low sanitary quality that a very dark, off-flavored sirup called "blackstrap" by maple producers was made from it.

On the basis of this collaborative study, the Associate Referee recommends that this method be adopted as official first action.

Acknowledgments

The Associate Referee acknowledges the able assistance in this work of V. G. Metzger, Eastern Regional Research Center, U.S. Department of Agriculture, Philadelphia, Pa. 19118, who carried out the statistical analysis of the collaborators' data and the cooperation of the following collaborators:

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W. R. Chesbro, University of New Hampshire, Durham, N.H.

A. Marin, New York Department of Agriculture, Albany, N.Y.

N. M. Vassau, Wisconsin Department of Agriculture, Madison, Wis.

P. J. Love, R. T. French Co., Rochester, N.Y.

J. T. Welch, Food and Drug Administration, Baltimore, Md.

B. E. Bartolomeo, Food and Drug Administration, Boston, Mass.

R. A. Bell, Eastern Regional Research Center, U.S. Department of Agriculture, Philadelphia, Pa.

This report of the Associate Referee was presented at the 87th Annual Meeting of the AOAC, Oct. 9–12, 1973, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D, and was adopted by the Association; *see* (1974) *JAOAC* **57**, 434.

This method will be designated as secs. **31.185–31.188** in the 12th Ed.

Table 1. Collaborator's data from study of maple sap resazurin test

Coll.	Bacterial counts/ml sap producing positive color at times shown						
	30 min	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
				L Culture			
1	9.1×10^7 3.2×10^7	9.1×10^6 3.2×10^6 9.4×10^6		9.1×10^5 9.4×10^5	3.2×10^5 9.4×10^4	3.2×10^4	
2	1.9×10^8 1.9×10^7 7.9×10^7	1.9×10^6 7.9×10^6 3.0×10^7	7.9×10^5 3.0×10^6		3.0×10^5		
3	3.4×10^8 1.0×10^8	3.4×10^7 3.6×10^7	1.0×10^7 3.6×10^6	1.0×10^6	3.6×10^5		
4		8.5×10^7	8.5×10^6 9.8×10^6		8.5×10^5 9.8×10^5	9.8×10^4	
5	4.3×10^8 1.5×10^8	4.3×10^7 6.1×10^7	1.5×10^7	4.3×10^6 6.1×10^6	1.5×10^6	6.1×10^5	
				S Culture			
6	1.7×10^7 8.2×10^6	1.7×10^6 2.1×10^6	8.2×10^5	1.7×10^5 2.1×10^5	8.2×10^4	2.1×10^4	
7	7.5×10^7	7.5×10^6 1.5×10^7	1.5×10^6 4.1×10^6	4.1×10^5	1.5×10^5 4.1×10^4		
8	2.0×10^8 2.0×10^7 8.0×10^7 3.6×10^7	8.0×10^6	2.0×10^6 3.6×10^6	8.0×10^5 3.6×10^5			
9	2.7×10^7 1.0×10^7	5.2×10^6	2.7×10^6 1.0×10^6	2.7×10 1.0×10^5 5.2×10^5	5.2×10^4		
10	1.1×10^7 2.9×10^7	4.3×10^6	2.9×10^6	1.1×10^6	4.3×10^5 2.9×10^5	4.3×10^4 1.1×10^5	
11	1.1×10^8	4.9×10^7	2.5×10^7	1.1×10^7	4.9×10^6	1.1×10^6 4.9×10^5 2.5×10^6	2.5×10^5
12	4.0×10^8		4.0×10^7 1.9×10^7	4.0×10^6 1.9×10^6 1.6×10^5	1.9×10^5 1.6×10^4		1.6×10^3

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References

(1) Kissinger, J. C. (1972) *JAOAC* **55**, 119–120

(2) Kissinger, J. C. (1969) *JAOAC* **52**, 714–716